

METHOD OF DETECTING MUTATION IN BASE SEQUENCE OF NUCLEIC ACID

BACKGROUND OF THE INVENTION

5 Field of the Invention

The present invention relates to a method of detecting mutation in the base sequence of nucleic acid including DNA (deoxyribonucleic acid) and RNA (ribonucleic acid).

Description of the Prior Art

10 It has been clarified that many cancers and genetic diseases are caused by mutation in the base sequence of DNA. The mutation in the base sequence is generally monobasic substitution. A number of methods have been proposed in the technical field of detecting such mutation in the base sequence.
15 Some of the methods are now illustrated.

1) DNA (RNA) Sequence:

The base sequence of a substance to be analyzed is directly analyzed and determined. Although this method is most reliable, it's disadvantage is the high cost required for a series of
20 operations. Further, a large-scale automation line is necessary for improving the throughput.

2) DNA Chip:

A number of oligonucleotides are fixed onto a glass surface and selectively hybridized with a substance to be
25 analyzed such as a DNA fragment for thereafter detecting a signal based on the hybridization, generally a fluorescent signal, and comparing the same with a normal one thereby estimating presence/absence of mutation in the sequence of the substance.

However, a DNA chip itself is extremely high-priced and
30 the number of oligonucleotides fixed onto the chip must be varied

with the substance, disadvantageously leading to a high cost.

3) SSCP (single strand conformation polymorphism)

Method:

Double stranded DNA (RNA) employed as a sample is converted to single stranded DNA for thereafter electrophoretically detecting the difference of stereochemical structure of the single stranded DNA which varies with the base sequence, thereby estimating presence/absence of mutation in the base sequence.

However, in this method, electrophoretic conditions must be studied every sample, and it is disadvantageously difficult to improve the throughput due to employment of gel electrophoresis.

4) DHPLC (denaturing high performance liquid chromatography) Method:

The DHPLC method which utilizes ion pair chromatography is disclosed in, for example, U.S. Patent No. 5795976. This method is now described with reference to Figs. 1(A) and 1(B).

(A) DNA fragments are subjected to PCR (polymerase chain reaction) amplification. It is assumed that normal DNA 2 having normal base sequence and mutational DNA 4 having mutational base sequence are mixed with each other as the DNA fragments (see Fig. 1(A)). The base sequence of the mutational DNA 4 is different from that of the normal DNA 2 in underlined portions.

(B) The normal DNA 2 and the mutational DNA 4 mixed with each other are thermally denatured into single stranded DNA, and thereafter the temperature is reduced for re-bonding the same. Consequently, homoduplexes 2a and 4a are formed by re-bonding source pair single strands while heteroduplexes 2b

and 4b are also formed by bonding single strands different from the source pair single strands (see Fig. 1(B)).

The homoduplexes 2a and 4a, which are identical in base sequence to the normal DNA 2 and the mutational DNA 4 respectively, form hydrogen bonds as to all base pairs. However, the heteroduplexes 2b and 4b have portions where corresponding bases are inappropriate, i.e., mismatching portions (underlined portions in Fig. 1(B)) forming no hydrogen bonds. Therefore, the homoduplexes 2a and 4a and the heteroduplexes 2b and 4b are different in stability from each other, and the melting temperature, at which 50 % of the total concentration of the double-stranded DNA is denatured to single stranded DNA, of the heteroduplexes 2b and 4b is reduced as compared with that of the homoduplexes 2a and 4a.

(C) The homoduplexes 2a and 4a and the heteroduplexes 2b and 4b are separated from each other by employing the principle of ion pair chromatography and setting a reversed phase column at the melting temperature of the heteroduplexes 2b and 4b. The DNA fragments which formed the heteroduplexes 2b and 4b cleave into single strands, and are detected faster than double strands. Therefore, when two detection peaks appear, it follows that the homoduplexes 2a and 4a and the heteroduplexes 2b and 4b are present in the PCR product, and hence it is understood that mutational ones have been present in the inspected sites of the DNA fragments before PCR amplification.

In the DHPLC method, mutation in the base sequence is inspected in units of exons. The exon is a part of the base sequence of DNA, ultimately forming information of protein biosynthesis as amino acid sequence, to be read and translated.

Assuming that the DHPLC method is employed for

simultaneously analyzing mutation of a plurality of exons (inspected sites), it is impossible to investigate the inspected site(s) having mutational base sequence. Therefore, in the DHPLC method, only mutation in the base sequence of one inspected site can be determined by single analysis. Therefore, in order to inspect mutation in a plurality of types of inspected sites, a series of operations of heating, re-bonding and analysis must be performed for each inspected site, disadvantageously leading to increase of the time and the cost required for the analysis.

SUMMARY OF THE INVENTION

Accordingly, an object of the present invention is to provide a method of detecting mutation in the base sequence of nucleic acid capable of discriminating and inspecting mutation in the base sequence of a plurality of types of inspected sites by performing a series of operations of re-bonding and analysis only once.

According to the present invention, a method of detecting mutation in the base sequence of nucleic acid includes the following steps (A) and (B):

(A) a bonding step of hybridizing an object of analysis consisting of nucleic acid or a nucleic acid fragment including a plurality of inspected sites to be subjected to inspection of mutation in the base sequence with a plurality of types of oligonucleotides having base sequence complementary to any of the inspected sites having normal base sequence and labeled to be discriminable from each other for forming duplexes, and

(B) a detection step of employing an ion pair chromatograph comprising a reversed phase column serving as a

separation column and a detector capable of discriminating and detecting the labeled oligonucleotides and setting the separation column at a temperature causing difference in stability between hetero- and homoduplexes included in the duplexes for analyzing the object of analysis.

In more detail, first, an object of analysis including a plurality of inspected sites is prepared. If the quantity of the object of analysis is small, it is preferable to amplify the object of analysis. An exemplary preferable amplification step is a PCR step. In order to suppress the cost for PCT reaction, the PCR step is preferably carried out only once.

A plurality of oligonucleotides having base sequence complementary to any of a plurality of types of inspected sites having normal base sequence and labeled to be discriminable from each other are prepared. While radioisotopes can be used as labels, preferable labeling materials are fluorescent materials. Oligonucleotides labeled with fluorescent materials are referred to as fluorescent oligonucleotides. The fluorescent oligonucleotides can be readily discriminated from each other by fluorescence spectra specific to the fluorescent materials. Description is made with reference to the fluorescent oligonucleotides.

The fluorescent oligonucleotides are hybridized with corresponding ones of the inspected sites of the object of analysis. In this hybridization, the object of analysis mixed with the fluorescent oligonucleotides is thermally denatured into single stranded DNA, and the temperature is thereafter reduced for bonding the single stranded DNA of the object of analysis with the fluorescent oligonucleotides. At this time, a homoduplex is formed in an inspected site having normal base

sequence while a heteroduplex is formed in an inspected site having mutational base sequence.

In the detection step, utilizing an ion pair chromatograph comprising a reversed phase column serving as a separation column and a detector capable of discriminating and detecting labels (fluorescent materials in this example) and setting the separation column at a temperature causing difference in stability between hetero- and homoduplexes, the object of analysis hybridized with the fluorescent oligonucleotides is introduced into the separation column along with a mobile phase mixed with an ion pair reagent. In the column, heteroduplexes are dissociated in a higher ratio than homoduplexes. Since dissociated fluorescent oligonucleotides elute in advance of hybridized fluorescent oligonucleotides, the fluorescent oligonucleotides having formed heteroduplexes elute in advance. The term "temperature causing difference in stability between hetero- and homoduplexes" stands for a temperature at which hetero- and homoduplexes are denatured and dissociated in different ratios, such as the melting temperature of the heteroduplexes or a temperature around the same.

A chromatogram of labels obtained through the detection step is observed for determining an inspected site corresponding to a label having a single peak as non-mutational while determining an inspected site corresponding to a label having two peaks as mutational. Thus, it is possible to recognize an inspected site forming a heteroduplex, and hence presence/absence of mutation in the base sequence can be investigated as to a plurality of inspected sites by single analysis.

The foregoing and other objects, features, aspects and advantages of the present invention will become more apparent

from the following detailed description of the present invention when taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figs. 1(A) and 1(B) are diagrams for illustrating a DHPLC method;

Fig. 2 is a schematic passage structural diagram showing an exemplary ion pair chromatograph;

10 Figs. 3(A) to 3(C) are diagrams for illustrating an embodiment of the present invention; and

Fig. 4 is a waveform diagram showing a chromatogram in the embodiment.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

15 Fig. 2 is a schematic passage structural diagram showing an exemplary ion pair chromatograph employed for the present invention.

20 A mobile phase is an acetonitrile solution containing triethylamine serving as an ion pair reagent. A gradient elution apparatus 1 supplies the mobile phase while varying the acetonitrile concentration thereof.

25 The gradient elution apparatus 1 is connected with a feed pump 3 feeding the mobile phase to a separation column 7. The separation column 7 is a reversed phase column. An exemplary reversed phase column has an internal surface formed by a nonporous material such as a nonporous polymer or nonporous silica, which is modified with an alkyl group such as an octadecyl group having 18 carbons connected in a straight-chain manner. Another exemplary reversed phase column is charged with a filler
30 which has a base material of a nonporous material such as a

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nonporous polymer or nonporous silica bonding an octadecyl group therewith.

A mobile phase passage between the feed pump 3 and the separation column 7 is provided with an injector 5 injecting a sample solution into the mobile phase passage. A column oven 9 adjusting the column temperature is provided around the separation column 7.

An elution side of the separation column 7 is connected to a detector 11 detecting an eluting component. The detector 11 is formed by that capable of discriminating a plurality of fluorescent materials such as three types of fluorescent materials F1, F2 and F3 that have different fluorescence spectral characteristics.

A fraction collector 13 fractioning an eluent on the basis of an output of the detector 11 is provided downstream the detector 11.

Figs. 3(A), 3(B) and 3(C) are diagrams for illustrating an embodiment of a method of detecting mutation in the base sequence of nucleic acid according to the present invention. This embodiment shall now be described with reference to Figs. 2 and 3(A) to 3(C).

A DNA fragment (object of analysis) containing a plurality of exons is subjected to PCR amplification. In this example, both normal DNA 15 and mutational DNA 17 are present as objects of analysis (see Fig. 3(A)). Referring to Figs. 3(A) to 3(C), symbols A, C, G and T denote adenine, cytosine, guanine and thymine respectively. Three exons 15a, 15b and 15c to be inspected are present in the normal DNA 15. Three exons 17a, 17b and 17c to be inspected are present in the mutational DNA 17, and it is assumed that the exons 17a and 17c have mutational

base sequence (underlined portions in Fig. 3(A)) as compared with the exons 15a and 15c.

A plurality of types of oligonucleotides (fluorescent oligonucleotides) 19a, 19b and 19c having base sequence
 5 complementary to the sequence of first chains forming the exons 15a, 15b and 15c having normal base sequence and labeled with the fluorescent materials F1, F2 and F3 respectively are prepared (see Fig. 3(B)). The oligonucleotides to be prepared may not
 10 correspond to all exons, but may correspond to only portions of the exons to be inspected. When inspecting mutation in the base sequence of the same exons as to a number of samples, the cost can be reduced by previously preparing a large quantity of fluorescent oligonucleotides.

The normal DNA 15 and the mutational DNA 17 subjected
 15 to PCR amplification and the fluorescent oligonucleotides 19a, 19b and 19c are mixed with each other in a solution, which in turn is heated under a temperature condition of, for example, 95 °C for 10 seconds to thermally denature and dissociate the normal DNA 15 and the mutational DNA 17 and thereafter
 20 maintaining the same at a temperature of 60 °C for 30 minutes for preparing a sample solution. Thus, the fluorescent oligonucleotides 19a, 19b and 19c are hybridized with the first chains of the corresponding exons 15a, 15b, 15c, 17a, 17b and 17c respectively, for forming homoduplexes 21a, 21b and 21c in a
 25 first chain 21 of the normal DNA 15 while forming heteroduplexes 23a and 23c and a homoduplex 23b in a first chain 23 of the mutational DNA 17 (see Fig. 3(C)). Bases shown with underlines in Fig. 3(C) mismatch in the heteroduplexes 23a and 23c, and hence the heteroduplex 23a has a lower melting temperature
 30 than the homoduplex 21a and the heteroduplex 23c has a lower

melting temperature than the homoduplex 21c.

The fluorescent oligonucleotides 19a, 19b and 19c forming no duplexes are removed from the sample solution, which in turn is thereafter analyzed with the ion pair chromatograph shown in Fig. 2.

The separation column 7 is adjusted to the melting temperature of the heteroduplexes 23a and 23c with the column oven 9. The feed pump 3 feeds the acetonitrile solution containing triethylamine to the separation column 7 as the mobile phase while adjusting the concentration of acetonitrile by the gradient elution apparatus 1. The sample solution is injected from the injector 5, mixed with triethylamine and introduced into the separation column 7. When the sample solution is mixed with triethylamine, triethylamine is coordinately bonded to phosphoric acid groups of the homoduplexes 21a, 21b, 21c and 23b and the heteroduplexes 23a and 23c contained in the sample solution, to improve hydrophobicity of these portions.

When the sample solution is introduced into the separation column 7 in this state, the heteroduplexes 23a and 23c are dissociated in the separation column 7 in a higher ratio than the homoduplexes 21a, 21b, 21c and 23b since the separation column 7 is adjusted to the melting temperature of the heteroduplexes 23a and 23c. Retention power of the separation column 7 for the labeled oligonucleotides 19a and 19c dissociated from the chain 23 is so reduced that the oligonucleotides 19a and 19c elute in advance of the hybridized labeled oligonucleotides 19a, 19b and 19c.

Fig. 4 is a waveform diagram showing a chromatogram in this embodiment. Referring to Fig. 4, the vertical axis shows intensity of fluorescence, and the horizontal axis shows retention

time.

Two detected peaks 27a and 29a appear on a detected waveform 25a of an F1 fluorescent channel, a single detected peak 27a appears on a detected waveform 25b of an F2
5 fluorescent channel, and two detected peaks 27c and 29c appear on a detected waveform 25c of an F3 fluorescent channel. Each of the detected waveforms 25a and 25c has two detected peaks since those of the labeled oligonucleotides 19a and 19c forming the heteroduplexes 23a and 23c have eluted in advance. In
10 other words, the detected peaks 27a, 29a, 27b, 27c and 29c show the presence of the homoduplex 21a (including the non-dissociated heteroduplex 23a), the heteroduplex 23a, the homoduplexes 21b and 23b, the homoduplex 21c (including the non-dissociated heteroduplex 23c) and the heteroduplex 23c
15 respectively.

Thus, it is understood that there has been mutational base sequence in the inspected sites corresponding to the exons 15a and 17a and the exons 15c and 17c of the object of analysis before the PCR amplification.

20 The present invention is not restricted to the aforementioned embodiment, and the structures of the ion chromatograph and the reversed phase column, the mobile phase and the ion pair reagent are not restricted to those in this embodiment either.

25 While mutation in the base sequence is inspected as to three inspected sites present in the same DNA fragment in the aforementioned embodiment, the object of analysis in the present invention is not restricted to this but may also be the overall nucleic acid including a plurality of types of inspected sites of
30 base sequence, or that prepared by mixing a plurality of nucleic

1990 1991 1992 1993 1994 1995 1996 1997 1998 1999 2000 2001 2002 2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014 2015 2016 2017 2018 2019 2020 2021 2022 2023 2024 2025 2026 2027 2028 2029 2030 2031 2032 2033 2034 2035 2036 2037 2038 2039 2040 2041 2042 2043 2044 2045 2046 2047 2048 2049 2050 2051 2052 2053 2054 2055 2056 2057 2058 2059 2060 2061 2062 2063 2064 2065 2066 2067 2068 2069 2070 2071 2072 2073 2074 2075 2076 2077 2078 2079 2080 2081 2082 2083 2084 2085 2086 2087 2088 2089 2090 2091 2092 2093 2094 2095 2096 2097 2098 2099 2100 2101 2102 2103 2104 2105 2106 2107 2108 2109 2110 2111 2112 2113 2114 2115 2116 2117 2118 2119 2120 2121 2122 2123 2124 2125 2126 2127 2128 2129 2130 2131 2132 2133 2134 2135 2136 2137 2138 2139 2140 2141 2142 2143 2144 2145 2146 2147 2148 2149 2150 2151 2152 2153 2154 2155 2156 2157 2158 2159 2160 2161 2162 2163 2164 2165 2166 2167 2168 2169 2170 2171 2172 2173 2174 2175 2176 2177 2178 2179 2180 2181 2182 2183 2184 2185 2186 2187 2188 2189 2190 2191 2192 2193 2194 2195 2196 2197 2198 2199 2200 2201 2202 2203 2204 2205 2206 2207 2208 2209 2210 2211 2212 2213 2214 2215 2216 2217 2218 2219 2220 2221 2222 2223 2224 2225 2226 2227 2228 2229 2230 2231 2232 2233 2234 2235 2236 2237 2238 2239 2240 2241 2242 2243 2244 2245 2246 2247 2248 2249 2250 2251 2252 2253 2254 2255 2256 2257 2258 2259 2260 2261 2262 2263 2264 2265 2266 2267 2268 2269 2270 2271 2272 2273 2274 2275 2276 2277 2278 2279 2280 2281 2282 2283 2284 2285 2286 2287 2288 2289 2290 2291 2292 2293 2294 2295 2296 2297 2298 2299 2300 2301 2302 2303 2304 2305 2306 2307 2308 2309 2310 2311 2312 2313 2314 2315 2316 2317 2318 2319 2320 2321 2322 2323 2324 2325 2326 2327 2328 2329 2330 2331 2332 2333 2334 2335 2336 2337 2338 2339 2340 2341 2342 2343 2344 2345 2346 2347 2348 2349 2350 2351 2352 2353 2354 2355 2356 2357 2358 2359 2360 2361 2362 2363 2364 2365 2366 2367 2368 2369 2370 2371 2372 2373 2374 2375 2376 2377 2378 2379 2380 2381 2382 2383 2384 2385 2386 2387 2388 2389 2390 2391 2392 2393 2394 2395 2396 2397 2398 2399 2400 2401 2402 2403 2404 2405 2406 2407 2408 2409 2410 2411 2412 2413 2414 2415 2416 2417 2418 2419 2420 2421 2422 2423 2424 2425 2426 2427 2428 2429 2430 2431 2432 2433 2434 2435 2436 2437 2438 2439 2440 2441 2442 2443 2444 2445 2446 2447 2448 2449 2450 2451 2452 2453 2454 2455 2456 2457 2458 2459 2460 2461 2462 2463 2464 2465 2466 2467 2468 2469 2470 2471 2472 2473 2474 2475 2476 2477 2478 2479 2480 2481 2482 2483 2484 2485 2486 2487 2488 2489 2490 2491 2492 2493 2494 2495 2496 2497 2498 2499 2500 2501 2502 2503 2504 2505 2506 2507 2508 2509 2510 2511 2512 2513 2514 2515 2516 2517 2518 2519 2520 2521 2522 2523 2524 2525 2526 2527 2528 2529 2530 2531 2532 2533 2534 2535 2536 2537 2538 2539 2540 2541 2542 2543 2544 2545 2546 2547 2548 2549 2550 2551 2552 2553 2554 2555 2556 2557 2558 2559 2560 2561 2562 2563 2564 2565 2566 2567 2568 2569 2570 2571 2572 2573 2574 2575 2576 2577 2578 2579 2580 2581 2582 2583 2584 2585 2586 2587 2588 2589 2590 2591 2592 2593 2594 2595 2596 2597 2598 2599 2600 2601 2602 2603 2604 2605 2606 2607 2608 2609 2610 2611 2612 2613 2614 2615 2616 2617 2618 2619 2620 2621 2622 2623 2624 2625 2626 2627 2628 2629 2630 2631 2632 2633 2634 2635 2636 2637 2638 2639 2640 2641 2642 2643 2644 2645 2646 2647 2648 2649 2650 2651 2652 2653 2654 2655 2656 2657 2658 2659 2660 2661 2662 2663 2664 2665 2666 2667 2668 2669 2670 2671 2672 2673 2674 2675 2676 2677 2678 2679 2680 2681 2682 2683 2684 2685 2686 2687 2688 2689 2690 2691 2692 2693 2694 2695 2696 2697 2698 2699 2700 2701 2702 2703 2704 2705 2706 2707 2708 2709 2710 2711 2712 2713 2714 2715 2716 2717 2718 2719 2720 2721 2722 2723 2724 2725 2726 2727 2728 2729 2730 2731 2732 2733 2734 2735 2736 2737 2738 2739 2740 2741 2742 2743 2744 2745 2746 2747 2748 2749 2750 2751 2752 2753 2754 2755 2756 2757 2758 2759 2760 2761 2762 2763 2764 2765 2766 2767 2768 2769 2770 2771 2772 2773 2774 2775 2776 2777 2778 2779 2780 2781 2782 2783 2784 2785 2786 2787 2788 2789 2790 2791 2792 2793 2794 2795 2796 2797 2798 2799 2800 2801 2802 2803 2804 2805 2806 2807 2808

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